30 minutes at 70° C. Wash the spore suspension three times with 25 to 50 milliliters of sterile distilled water. Resuspend the organisms in 50 to 70 milliliters of sterile distilled water and heat-shock again for 30 minutes at 70° C. Use test plates to assure the viability of the spores and to determine the amount of spore suspension to be added to each 100 milliliters of agar. Maintain the spore suspension under refrigeration.

(4) [Reserved]

(5) *Method 5.* Maintain the test organisms in 100–milliliter quantities of nutrient broth—Medium 3 as described in § 436.102(b)(3). For the test prepare a fresh subculture by transferring a loopful of the stock culture to 100 milliliters of the same nutrient broth and incubate for 16 to 18 hours at 37° C. Store this broth culture under refrigeration.

(6) *Method 6.* Maintain the test organisms on agar slants containing 10 milliliters of the medium specified in paragraph (a) of this section. Incubate the slants at 32° C.–35° C. for 24 hours. Inoculate 100 milliliters of nutrient broth—Medium 13 as described in § 436.102(b)(13). Incubate for 16 to 18 hours at 37° C. Proceed as directed in paragraph (b)(1)(ii) of this section.

(7) *Method 7.* Proceed as directed in paragraph (b)(1) of this section, except incubate the slants at 30° C. for 24 hours and incubate the Roux bottle at 30° C. for 48 hours.

(8) *Method 8.* Maintain organisms on agar slants containing 10 milliliters of the appropriate medium and transfer to a fresh slant about once a week. Incubate the slants at 37° C for 48 hours. Using 3 milliliters of sterile U.S.P. saline T.S., wash the growth from the agar slant into a 500–milliliter Erlenmeyer flask containing 100 milliliters of medium 34, as described in § 436.102(b) (34), and 50 grams of glass beads. Agitate the culture by rotation at a speed of 130 cycles per minute and a radius of 3.5 centimeters at 27° C for 5 days. Determine the amount of suspension to be added to each 100 milliliters of agar by the use of test plates. Store the test organism suspension under refrigeration.

(9) *Method 9.* Proceed as directed in paragraph (b)(1) of this section, except incubate the slant and Roux bottle at 37° C and wash the resulting growth from the agar surface with 50 milliliters of Medium 37 as described in § 436.102(b)(37).

[39 FR 18944, May 30, 1974, as amended at 40 FR 52004, Nov. 7, 1975; 42 FR 14092, Mar. 15, 1977; 42 FR 18058, Apr. 5, 1977; 44 FR 10378, Feb. 20, 1979; 47 FR 22514, May 25, 1982; 47 FR 27552, June 25, 1982]

§ 436.104 Penicillin activity.

Use penicillin-free equipment and glassware.

(a) *Preparation of inoculated plates.* Proceed as directed in § 436.105(a), using 10 milliliters of medium 1 for the base layer. For the seed layer, use 4 milliliters of medium 4, inoculated with the amount of test organism C which gave the clearest, sharpest zones of inhibition measuring 17 to 21 millimeters in diameter when standardized as described in § 436.103(b)(1)(ii). Use the plates the same day they are prepared.

(b) *Preparation of working standard stock solutions and standard response lines solutions.* Proceed as directed for penicillin G in § 436.105(b), except dilute the working standard stock solution to a final concentration of 100 units of penicillin G per milliliter and use the following final concentrations for the standard response line: 0.005, 0.0125, 0.025, 0.050, 0.100, and 0.200 unit of penicillin G per milliliter. The 0.050 unit of penicillin G–per–milliliter solution is the reference concentration of the assay.

(c) *Sample preparation.* Dissolve 1.0 gram of the sample in sufficient distilled water to make 18 milliliters. Filter if not clear. Transfer 9.0 milliliters to a separatory funnel, and add 20 milliliters of amyl acetate. Add 1 milliliter of 10 percent potassium phosphate buffer, pH 2.5 (solution 11 as described in § 436.101), shake, allow to separate, and draw off the aqueous layer into a second separatory funnel. Check the pH of the aqueous solution with pH paper, and readjust with concentrated hydrochloric acid if the pH is three or above. Extract again with 20 milliliters or amyl acetate, discard the aqueous phase, and combine the amyl acetate extracts. Wash the extracts with 10 milliliters of 1 percent potassium phosphate buffer, pH 2.5, and discard the

buffer wash. Extract the penicillin from the amyl acetate with a 10–milliliter aliquot of 1 percent potassium phosphate buffer, pH 6.0 (solution 1 as described in § 436.101). This is the assay solution.

(d) *Procedure for assay.* For the standard response line, use a total of 15 plates (three plates for each response line solution, except the reference concentration solution, which is included on each plate). On each set of three plates, fill three alternate cylinders with the reference concentration solution and the other three cylinders with the concentration of the response line under test. Thus, there will be 45 reference concentration zones of inhibition and nine zones of inhibition for each of the other concentrations of the response line. Treat a portion of the sample solution (2 to 5 milliliters) with 0.1 milliliter of penicillinase solution and incubate at 37° C. for 1 hour. For each sample tested, use three plates. On each plate fill two cylinders with the 0.050 unit of penicillin G per milliliter standard, two cylinders with the untreated sample, and two cylinders with the penicillinase-treated sample. Incubate all plates, including those of the standard response line, overnight at 30° C. A zone of inhibition with the untreated sample and no zone with the penicillinase-treated sample are a positive test for penicillin. If a positive test is obtained, measure the diameters of the zones of inhibition using an appropriate measuring device such as a millimeter rule, calipers, or an optical projector.

(e) *Estimation of penicillin G activity.* To prepare the standard response line, average the diameters of the standard reference concentration and average the diameters of the standard response line concentration tested for each set of three plates. Average also all 45 diameters of the reference concentration. The average of the 45 diameters of the reference concentration is the correction point of the response line. Correct the average diameter obtained for each concentration to the figure it would be if the average reference concentration diameter for that set of three plates were the same as the correction point. Thus, if in correcting the 0.025 penicillin G concentration, the average of the 45 readings of the 0.050 unit of penicillin G–per–milliliter concentration is 18.5 millimeters and the average of the 0.050 unit of pencillin G–per milliliter concentration of this set of three plates is 18.3 millimeters, the correction is +0.2 millimeters. If the average reading of the 0.025 unit of pencillin G–per–milliliter concentration of these same three plates is 15.5 millimeters, the corrected value is 15.7 millimeters. Plot these corrected values, including the average of the 0.050 unit of penicillin G-per-milliliter concentration, on semilogarithmic graph paper using the pencillin concentration in units per milliliter on the logarithmic scale and the diameter of the zone of inhibition on the arithmetic scale. Draw the line of best fit through these points. To estimate the sample potency, average the zone diameters of the standard and the zone diameters of the sample on the three plates used. If the average zone diameter of the sample is lower than that of the standard, subtract the difference between them from the reference concentration diameter of the standard response line. From the response line, read the concentrations corresponding to these corrected values of zone diameters. Multiply the concentration by the dilution factor to obtain the units of penicillin G per sample size tested.

[39 FR 18944, May 30, 1974, as amended at 41 FR 34743, Apr. 17, 1976]

§ 436.105 Microbiological agar diffusion assay.

Using the sample solution prepared as described in the section for the particular antibiotic to be tested, proceed as described in paragraphs (a), (b), (c), and (d) of this section.

(a) *Preparation of inoculated plates.* For each antibiotic listed in the table in this paragraph, select the media (as listed by medium number in § 436.102(b)), the amount of media to be used in the base and seed layers, the test organism (as listed in § 436.103(a)), and the suggested inoculum and prepare the inoculated plates as follows: Prepare the base layer by adding the appropriate amount of melted agar to each Petri dish (nominal dimensions 20 by 100 millimeters). Distribute the agar